Summary report for contract number FIGH-CT-1999-0004

Chernobyl, an Integrated, Pan-European Study: Morphology, Oncogenes, DNA Repair and Outcome in Radiation Carcinogenesis (CHIPS)

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Introduction

The availability of paired normal tissue/thyroid carcinoma samples from a population where the tumours can be shown to due to exposure to radiation (those exposed in childhood to radioactive fallout from the Chernobyl nuclear disaster) presents a unique opportunity to unravel the mechanisms involved in radiation associated carcinogenesis. The objective of this study is to investigate the link between exposure of children to radiation, the subsequent development of tumours and how their morphology, molecular and cell biology influence clinical outcome.

The project is an integrated approach involving 5 leading European centres. Samples of the same tumours will be studied by the 5 different centres to determine tumour morphology and type; the degree of variation within the tumour, including the variation of the proportion of tumour cells in cycle using antibodies to novel DNA replication-associated peptides; the genes involved in the carcinogenic process, using DNA chip technology; specific studies of the pathways associated with one oncogene (ret) known to be linked to the tumour type involved; and studies of novel gene rearrangements using FISH technology and studies of the control of DNA replication

in tumour and normal cells using a novel in vitro cell free assay. Using different approaches on the same tumour provides a very powerful tool – for example the identification of tumour specific RNAs by DNA chip technology will be correlated with the identification of tumour specific peptides in the cell free assay for DNA replication and with the peptides produced by known or novel tumour specific translocations. By using the same tumour/normal pairs in these studies, integrating the results from the different centres and studies and correlating these with detailed morphological studies and patient details including evidence on tumour aggressiveness and recurrence, our understanding of the link between radiation exposure and cancer development will be increased, and provide evidence which will inform decisions on radiation protection and on clinical management of patients with radiation associated cancers.

The key aims of this project are to use this unique opportunity to study in detail a human tumour for which the aetiological agent is known. This will:

- provide a better assessment of risk following exposure to radioactive fallout from an accident in a nuclear power plant, in particular risk to particular cohorts. This would help to decide appropriate population screening procedures and therefore improve healthcare economic planning in the event of a future accident, as well indicating appropriate prophylactic measures
- provide possible prognostic markers which may define appropriate levels of healthcare surveillance of affected individuals
- indicate new therapeutic avenues, which may be of use in other human cancers, both radiation related or due to other causes
- lead to a better understanding of the relationship between radiation exposure, gene defects and DNA repair in the mechanistic changes that interact with specific cellular pathways and lead to carcinogenesis.

Summary of results

Detailed pathological analysis was performed on 181 thyroid cancers and follicular adenomas, the majority of which were provided by the Chernobyl Tissue Bank (www.chernobyltissuebank.com) The details are given in Table 1.

Papillary carcinomas (PTCs) are not monomorphic, and our earlier studies showed that different rearrangements of the ret oncogene correlated with differing morphological patterns of growth (1). The majority of cases of the 134 PTC used in this study were of mixed phenotype showing elements of both solid and follicular or papillary architecture (49%). Smaller proportions are of purely papillary (13%), purely follicular (15%) purely solid (9%) architecture. The smallest proportion is of mixed papillary/follicular architecture (6%). There has been a change with time in the morphological pattern of growth observed in post Chernobyl PTC. Shorter latency papillary cancers appear to be dominated by a solid growth pattern, whereas the later papillary cancers appear to show a more follicular or papillary architecture (2).

Table 1: Pathology of cases analysed through the CHIPS project. Detailed pathological analysis is provided in the annex to this report.

Pathology	number of cases
PTC	134
FC	6
MTC	2
WDCA NOS	5
PDC	1
FA	28
Total	181

We studied the expression of two markers of proliferation (ki67 and mcm2) in 62 papillary carcinomas and showed that there was no significant correlation between the numbers of epithelial cells positive for ki67 or mcm2 or the ratio of mcm2/ki67 with either size of tumour, age at exposure to radiation or sex. There was however, a significant association ($p<0.001$ Mann Whitney test) with both ki67 and mcm2 and the latent period (lower proportion of positive cells associated with longer latency) and a significant correlation ($p<0.001$ Mann Whitney test) with the proportion of positive ki67 in the centre of the tumour and regional metastasis. We were unable to show a correlation between degree of positivity of proliferation and rearrangement of the ret oncogene.

We also compared gal-3 mRNA levels by quantitative PCR (QPCR) and protein localisation by immunocytochemistry (ICC) in paired samples of tumour and normal tissue from 50 cases of PTC and 14 cases of cellular follicular adenoma (FA) from Ukraine. PTCs showed elevated levels of Gal-3 mRNA on QPCR relative to normal tissue (Wilcoxon test $p< 1.209 \times 10^{-6}$), and predominantly universally positive staining in the cytoplasm of the follicular cells comprising the tumour. Nuclear positivity was observed in nearly all PTCs, but this was not marked. Gal-3 ICC showed positive staining only in endothelial cells in the normal thyroid tissue, whether derived from patients with PTC or FA. Endothelial staining was not seen within PTCs. FAs, in contrast to PTCs showed a lower level of gal-3 RNA on QPCR compared to paired normal tissue (Wilcoxon test $p< 3.209 \times 10^{-5}$). On ICC, only two of the adenomas showed localisation of gal-3 to the follicular cells; the majority showed negative follicular epithelium, but marked positivity of endothelial cytoplasm within the tumour. In two FAs, areas of tumour with nuclear and cytoplasmic changes similar to that seen in PTCs showed strong cytoplasmic positivity for gal-3 protein. These results suggest that gal-3 may be a useful marker for distinguishing PTC from follicular tumours, but not a suitable for tool in preoperative diagnosis for determining malignancy in follicular tumours. The differences in endothelial positivity between PTCs and FAs are intriguing and warrant further investigation.

Ret expression was examined in 165 tumours, (see table 2 for results).

Table 2 Expression of the ret oncogene

Pathology	number of cases examined (see above)	positive for ret TK expression*
PTC	124	31 (25%)
FC	5	0
MTC	2	2 (100%)
WDCA NOS	4	0
PDC	1	0
FA	27	0
WDT	2	0
Total	165	33

NB all PTCs were negative for expression of the extracellular (EC) domain of the ret gene, indicating activation by translocation. The two MTCs studied were positive for both TK and EC, indicating presence of the full length transcript for ret.

We also examined ret rearrangement by FISH in 32 of these cases (in tumour and normal pairs), and identified the rearrangements (where present) using rearrangement specific RT-PCR. 23 of 32 post Chernobyl cases (72 %) showed RET rearrangements diagnosed by FISH interphase analysis. The highest frequency of rearranged cells after FISH interphase analysis was 46%. Intriguingly, none of the tumours showed 100% rearranged cells. It became obvious that some cases showed a clustering of rearranged cells on LSM evaluation within individual areas. A statistical test for homogeneity (distribution homogeneity test) was performed and individual cases that show either a homo or heterogeneous distribution of nuclei positive for rearrangement were identified.. Statistically a non-homogeneous distribution can be randomly expected within one to two cases out of 32 cases. In our analysis nine cases showed a p-value of < 0.05 indicating a non-homogeneous distribution and, therefore, subclones of tumor cells either with or without RET rearrangement.

A comparison between the different approaches used in this study is given in Figure 3. All six cases showing an elevated expression of the TK domain of RET and a RET/PTC rearrangement (cases 24 to 29) exhibited rearranged cells in FISH analysis above the cut-off level too (subgroup 1). Most of the cases belonging to this subgroup showed a large fraction of FISH positive cells. A further 16 cases (cases 1 to 16) showed neither overexpression of the RET TK nor positivity for PTC1 or 3 on rearrangement-specific RT-PCR. However, twelve of these 16 cases exhibited a frequency for FISH positive nuclei greater than 7.1 % (subgroup 2). Notably, most of these cases showed a fraction of FISH positive cells lower than samples of the first subgroup. It is likely that due to the clustering of RET/PTC positive cells the sensitivity of the RT-PCR was not high enough to detect the few rearranged cells. A third subgroup consisted of six cases (cases 18 to 23) that showed elevated expression of the TK domain of RET, but are missing RET/PTC rearrangements after RT-PCR analysis. Four of these cases exhibited split FISH signals in more than 7.1 % of cells analysed by interphase FISH. Thus, likely they harbour RET rearrangements other than H4/RET (RET/PTC1) and RFG/RET (RET/PTC3). One other case revealed a RET/PTC3 rearrangement after RT-PCR analysis and a significant number of rearranged cells (>7.1 %) after FISH analysis, but it failed to exhibit an elevated

expression of the TK domain of RET. Unfortunately there was insufficient material to examine this case further.

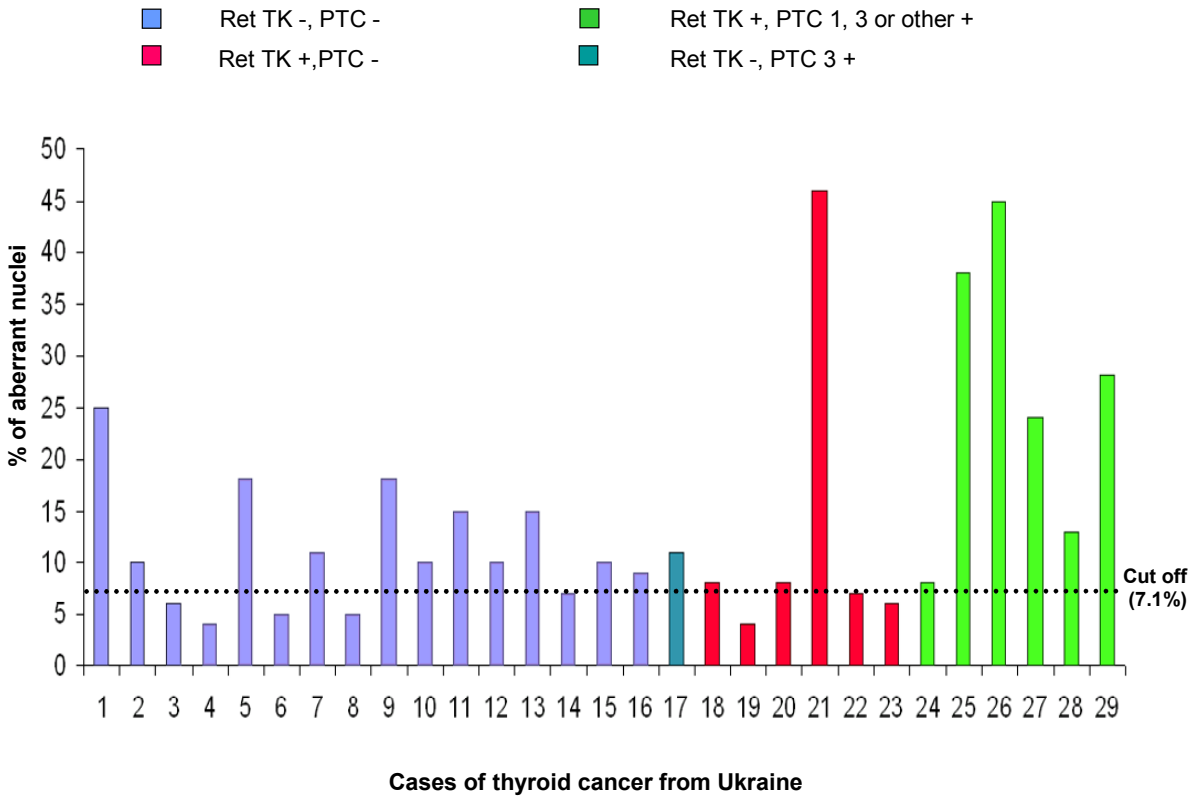

Figure 1: Comprehensive investigation of 29 cases of post-Chernobyl papillary thyroid carcinomas with three different approaches for the detection of RET rearrangements. Different colours of colums indicate various levels of agreement of results between RET/PTC- and RET-TK expression analysis. The columns represent the frequency of aberrant cells per cases detected by interphase FISH. For interpretation of FISH results a cut-off level of 7% is indicated. For full details of these results please see reference 3.

Gene expression profiling

cDNA array analysis was performed on 12 papillary cancers using the Micromax Human cDNA microarray system. The results were compared with 9 sporadic PTC, 13 autonomously functioning adenomas, and 1 multinodular goiter.

Our results show that 1) RNA profiles from sporadic and post-Chernobyl PTC did not identify a molecular signature specific for radiation-induced or non-radiation-induced PTC, on the basis of the expression of 2400 randomly selected genes; 2) pooled RNA extracts from sporadic and post-Chernobyl PTC show high correlation on the basis of 17 000 genes; and 3) sporadic and post-Chernobyl PTC pools are as strongly correlated as two independently derived PTC pools on the basis of 6425 genes. The similarity of gene expression in the two tumor groups is striking and demonstrates that both groups actually represent the same disease, i.e. there is no specific "radiation fingerprint" in the post-Chernobyl PTC. This suggests that the initial event in thyroid tumorigenesis (RET/PTC rearrangement or others) does not influence the phenotype

of the tumor. Our own preliminary studies correlating ret rearrangement with hierarchical clustering data from cDNA array support this Whatever this initial event, it is the same transduction pathway, the constitutive tyrosine kinase cascade, which is activated in all these tumors, i.e. the pathogenic mechanism is the same. Different levels of activity of this pathway could lead to different growth rates and phenotypes. For full details of these studies please see reference 4.

Cytogenetic analysis

Cell cultures were prepared from a total of 40 cases of thyroid cancer. Cytogenetic analysis using SKY revealed a clonal chromosomal aberration in 2/18 cases. CGH on paraffin sections of the primary tumours revealed that 67% (8/12) showed chromosomal imbalances. Please see reference 5 for more details.

The pathological data, which includes information on size of tumour, age at operation, age at exposure, latency, capsular and vascular invasion, distant and local metastases, the morphological data has been entered into the CHIPS project database, together with all the experimental data generated from the project. This has produced a very large amount of data which will enable correlations between morphological subtype, molecular biology and clinical presentation. The database will be analysed by a professional statistician and the overarching results published in the scientific literature. In addition, the information from the CHIPS data base which relates to cases supplied by the Chernobyl Tissue Bank, will be provided to the Coordinating Centre for that project for entry into that database and correlation with studies carried out by other research groups.

1: Santoro M, et al., (2000) Gene rearrangement and Chernobyl related thyroid cancers. Br J Cancer 82: 315-322

2: Williams ED et al., (2004) Thyroid Carcinoma after Chernobyl. Latent Period, Morphology and Aggressivity. Br J Cancer in press

3: Unger K, et al., (2004) Comprehensive evaluation of ret rearrangements in post Chernobyl thyroid cancers by FISH and RT-PCR indicates genetic heterogeneity and polyclonal tumor growth. Submitted

4: Detours V et al., (2004) Post Chernobyl thyroid papillary carcinomas have the same phneotype as sporadic PTC. Submitted

5: Richter H, et al (2004) Chromosomal inbalances in post Chernobyl thyroid Tumours. submitted